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The Patent Office
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1. Your reference — NCB / P20053GB

2. Pate *(The* — **9701245.4** — 22 JAN 1997

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — EISAI CO., LTD.
TOKKYOBU, KOISHIKAWA,
BUNKYO-KU, 4-6-10,
TOKYO, JAPAN, 112-88

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — JAPAN

4. Title of the invention — METHOD OF SCREENING COMPOUNDS

5. Name of your agent *(if you have one)* — KILBURN & STRODE
30 JOHN STREET
LONDON
WC1N 2DD

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Patents ADP number *(if you know it)*

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
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Description	9
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Signature K Wilburn Stride Date 22 JANUARY 1997

— WILBURN & STRODE

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PATENT
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METHOD OF SCREENING COMPOUNDS

Background of the Invention

This invention relates to apoptosis, which is associated with physiological or programmed cell death (PCD). Apoptosis occurs in embryonic development, hormone deprivation of endocrine or other hormone-dependent or sensitive cells, cells responding to mild thermal or metabolic stress, and normal tissue turnover. Compounds which affect PCD (either accelerating or inhibiting the process) are potentially useful as therapeutics to treat a wide range of medical disorders, including cancer, AIDS, autoimmune disorders such as rheumatoid arthritis, and neurodegenerative diseases such as multiple sclerosis.

Cell death in many (but not all) types of cells can be thought of as a three-step process. The first step is the transmission of information about the status of the cell from outside the cell to the cytoplasm, or from the cell membrane to the nucleus. This information may consist of the appearance of an apoptosis-inducing factor such as the Fas ligand, or the disappearance of a survival-promoting factor, such as nerve growth factor (for some types of neurons). The second step is gene transcription and translation into protein. This second step can be blocked, in some forms of apoptosis, by compounds such as actinomycin D or cycloheximide, compounds which block transcription or translation. In the third step, the effectors of cell death are activated; these effectors include (in many types of cells) cysteine proteases which cleave after aspartic acid residues; these agents are now termed caspases. Caspase inhibitors can interrupt the programmed cell death chain of processes, by blocking this third step.

Summary of the Invention

We have developed a novel method for screening potential apoptosis-affecting compounds in an intact animal. The method employs the steps of: a) providing an embryo of a species of osteichthes (preferably a zebrafish, *Danio rerio*), which is translucent or transparent (i.e., optically clear), b) contacting the test compound with the clear embryo, and c) visually observing the pattern or extent of cell death in the embryo.

Preferably, cells in the embryo undergoing programmed cell death are labelled *in vivo* for microscopic visualization.

The invention in part is based on our observation that cells in the zebrafish embryo undergo apoptosis during normal development. We have found that the dying cells can be identified by simply viewing the whole live embryo (e.g., using Nomarski optics, or by a vital stain such as acridine orange). Alternatively, the dying cells can then be viewed and analyzed histologically, by staining the entire embryo using a method (the TUNEL method, explained in detail below), which detects DNA in the process of fragmenting during cell death. The pattern of TUNEL-positive cells at about 24 hours of development is easily discernable and very reproducible. Our studies indicate that some of the dying cells are neurons, including Rohon-Beard sensory neurons, and thus the method provides an important tool for studying neuronal apoptosis.

Zebrafish are well suited for use in the method of the invention because of their rapid development, large brood size, external fertilization and, most importantly, the optical clarity and large size of their embryos. Because the embryo is clear, apoptotic cells can be detected under the light microscope as highly refractive bodies, or stained using vital dyes such as acridine orange.

The amount of normal cell death in the developing fish embryo, as in other vertebrate embryos, is relatively small at any point in time. According to another aspect of the invention, we have discovered that the amount of cell death in these embryos can be greatly increased by briefly exposing the live embryos to a protein kinase inhibitor such as staurosporine. Our experiments demonstrated that exposure of embryos to micromolar amounts of staurosporine produced large numbers of TUNEL-positive cells. The number of dying cells was so large that they could easily be observed by microscopy, even without TUNEL staining. Cell death was so extensive that entire structures, e.g., the caudal tail region, were affected in a manner which could be observed microspically.

Thus, generally, the invention features a method for obtaining any of a wide variety of information on cellular processes by (a) providing a test and a control zebrafish embryo, (b) subjecting the test embryo to test conditions, and (c) visually observing differences in cells of the test and control embryos; those differences result from the application of the test conditions. Any of a variety of test conditions can be employed, including mutagenesis, which can be carried out by any standard methods, e.g., radiation, or chemical agents.

Embryos can be treated with agents which inhibit apoptosis, e.g., caspase inhibitors, and the "saved" neurons can then be examined to determine whether they develop or function normally. Neural connectivity can also be observed using the system of the invention, as can cell, tissue, and development.

The invention can also be used to test compounds for the ability to affect expression of a gene whose expression ordinarily affects cell death. The method involves the steps of: (a) providing an osteichthes (preferably zebrafish) test embryo which is translucent or transparent, and in which the cell death-affecting gene is expressed; the gene either is one which is not normally expressed in the species of which the embryo is a member, or is normally expressed at lower levels, and is over-expressed in the embryo. The test compound is contacted with the test embryo, and changes in the pattern or extent of cell death in the embryo brought about by the compound indicate its affect on cell death. Compounds which inhibit cell death-blocking compounds are useful, e.g., as anti-tumor adjuvant therapeutics.

Any of the known genes which express proteins which inhibit or accelerate cell death can be used; one example is the bcl-2 gene, the overexpression of which can be expected to block apoptosis. The embryo can be caused to express or over-express the cell death-affecting gene either via a transgenesis (the gene is inserted into embryo by standard microinjection techniques), or cells which have been transfected *ex-vivo* with the gene can be transplanted into the embryo.

The invention offers ease of use compared to other vertebrate embryonic systems (such as rodent or avian), while the process of cell death is similar or identical. Thus, compounds identified according to the invention which are effective anti-apoptotic agents are likely to be effective in mammalian systems as well. A further advantage of the invention is that the embryos are treated while they are still alive and developing, and it is therefore possible to determine if cells that are prevented from dying develop normally, an important consideration in screening anti-cell death drug candidates.

Other features and advantages of the invention will be apparent from the detailed description thereof, and from the claims.

Detailed Description

Embryos

The embryos used in the methods of the invention preferably are clear and large enough for easy microscopic visualization . A number of osteichthes (bony fish) species are suitable, e.g., Medaka, Giant rerio. The preferred species is *Danio rerio*, the zebrafish, which has large, clear, easily-visualized embryos, and which reproduces in large numbers.

Cell Death Inhibition Assay

The assay is carried out as follows. Ten zebrafish embryos, at 90% epiboly (about 9 hours), are placed in a tank containing standard fish H_2O (60 mg Instant Ocean/liter distilled water) and varying concentrations (0.001-1000 μM) of test compound. The embryos are incubated with the test compound overnight (until they reach about 22 hours of development), and examined *in vitro* and then prepared as follows for histologic examination.

The embryos are either acridine orange labelled or dechorionated and fixed in 4% paraformaldehyde made up in PBS. They can then be viewed with Nomarski optics or processed for TUNEL.

Acridine Orange Staining

Embryos of different ages are collected and incubated for 15-20 min. In 5μg/ml acridine orange (Sigma). The embryos are then anaesthetized and observed under a microscope. Acridine orange-positive apoptotic cells are clearly visible under fluorescent illumination. They are then photographed and counted.

TUNEL Staining

The acronym TUNEL stands for terminal deoxynucleotide transferase (TdT) dUTP nick labelling, a method which detects DNA fragmentation which is characteristic of dying cells. In this method, terminal deoxynucleotide transferase DNA polymerases target the multitude of new 3'OH ends generated by DNA fragmentation in both early stage and morphologically identifiable nuclei and apoptotic bodies. TdT polymerases add digoxigenin-dUTP to the 3'OH ends of the PCD fragmented DNA, which can then be detected by anti-digoxigenin alkaline phosphase conjugate, and stained with substrate.

In summary, the TUNEL staining method is carried out as follows:

Embryos are fixed and washed in PBT buffer. They are then treated with proteinase K, washed, and postfixed in paraformaldehyde. They are rinsed, fixed in methanol/acetic acid, rinsed again, and then subjected to the terminal transferase reaction. Embryos are incubated with terminal deoxytransferase (TdT) using reagents and conditions provided in the Apoptosis Detection Kit supplied by Oncor, Inc. Enzyme incubation is overnight at 37 °. The reaction is stopped and the embryos are rinsed in PBT. For detection of labelled DNA, embryos are incubated in sheep anti-digoxigenin antibody conjugated to alkaline phosphatase. An alkaline phosphatase substrate X-phosphate/NBT in an appropriate buffer is added and incubation continues for 15 minutes. The reaction is stopped, embryos are fixed, cleared, mounted, and viewed with Nomarski optics.

The detailed TUNEL protocol is as follows:

Fixation

1. Dechorionate embryos and fix in 4% paraformaldehyde/PBS for 1 hr. at room temperature. Wash 3 x 5 min. In PBS. Embryos can be stored in methanol at 4° overnight.

Permeabilization

1. Rehydrate by careful washes in 75% methanol + 25% PBT (1 x PBS, 0.1% Tween 70); 50% methanol; 50% PBT; 75% methanol; 75% PBT for 5 min. each.

2. Wash 3 x for 5 min. In PBT.

3. Incubate embryos in Proteinase K (10 µg/ml in PBS at room temperature 20 min. For post 16 hr. Wash 2 x for a few seconds in PBT.

4. Postfix embryos in 4% paraformaldehyde/PBS for 20 min. at room temperature.

5. Wash 5 x 5 min. In PBT.

6. Postfix embryos for 10 minutes at -20C. With prechilled (-20°C) Ethanol:Acetate +2:1.

7. Wash 3 x 5 mins. In PBT at room temperature.

Terminal Transferase Reaction

1. Incubate embryos for 1 hr. at room temperature in 75 µl (1 drop) equilibration buffer, reaction buffer and TdT enzyme are provided in the ApopTag In situ Apoptosis Detection Kit-Peroxidase; Oncor, Inc. For preparation of working strength TdT enzyme, mix the reaction buffer (S7105) with the TdT enzyme (S7107)=2:1 and add Triton X 100 to a final concentration of 0.3%).

2. Take off as much equilibration buffer as possible and add small volume of working strength TdT enzyme (The reaction worked already with as little as 17 µl working strength TdT enzyme). Incubate overnight at 37°C.

Stop/Wash

1. Stop reaction by washing in working strength stop/wash buffer (prepare working strength stop/wash buffer by mixing 1 ml stop/wash buffer (S7100-4) with 17 ml distilled water) for 3 hours at 37°C.

2. Wash 3 x 5 mins. In PBT.

Detection

1. Block with 2 mg/ml BSA, 5% sheep serum in PBT for a minimum of 60 mins. (Or use 4% BSA, 5% non-fat dry milk, 10% horse serum).

2. Incubate embryos for 2 hours at room temperature (or overnight at 4°C.) In a 1/2000 dilution of preabsorbed sheep anti-digoxgenin-alkaline phosphatase conjugated Fab fragments.

3. Wash overnight with 2 mg/ml BSA in PBT with at least 4 changes of blocking buffer.

4. Equilibrate 3 x 5 min. in freshly prepared NTMT buffer (0.1 M Tris-HCl pH 9.5; 50 mM $MgCl_2$; 0.1M $NaCl_2$; 0.1% Tween 20).

5. Perform color reaction with X-phosphate/NBT in NTMT on shaker in dark (4.5 μl of 75 mg/ml NBT in dimethylformamide and 3.5 μl of 50 mg/ml X-phosphate in dimethylformaldehyde in 1 ml NTMT buffer) for 15 minutes.

6. Stop reaction with washes in PBT.

Clearing and Mounting

1. Fix embryos in 4% paraformaldehyde/PBS for 30 min. at room temperature.

2. Wash in PBT.

3. Clear and mount in glycerol - 30% - 50% - 70%.

4. Document with Nomarski optics.

Other staining methods which detect DNA fragmentation characteristic of dying cells can be used in place of TUNEL staining, e.g., propidium iodide or Hoechst 33342 dye incubation followed by examination under fluorescence optics to detect

condensed chromatin. Extraction of DNA is followed by resolution on gels to detect DNA "laddering" into nucleosome-sized fragments of about 180 bp.

Programmed Cell Death in Normal Embryos

TUNEL staining was used to identify the pattern of PCD occurring in various stages of normally-developing zebrafish. Embryos 30% epiboly to 24 hours were studied every hour, and embryos 24 hours to 48 hours were studied every two hours.

Diffuse PCD was observed in most regions during development, with concentrated regions of PCD which were localized spatially and temporally. The earliest PCD was detected in a few cells at 75% epiboly. Up to 12 somites, there is diffuse, seemingly random PCD, which then begins to concentrate toward the brain and tailbud. From 19 hours, a localized pattern of PCD was found in the lens and cornea of the eye, the otocyst, the cloacal opening, the olfactory placode, and portions of the nervous system, including the dorsoventral spinal cord.

Diffuse apoptosis is believed to occur amidst tightly packed cells in order to allow their free movement during periods of gradual morphogenesis. Highly localized concentrations of PCD may permit more radical morphogenesis. For example, localized cell death in the nervous system may clear the way for outgrowing axons; concentrated PCD in the olfactory placode coincides with the time that axons are exiting the placode and growing toward the telencephalon.

PCD Increased with Kinase Inhibitors

The protein kinase inhibitor staurosporine is used at a concentration of between 10 μM and 100 μM. Embryos at the 22 hour stage are incubated for 120 minutes, washed in PBS, and either labelled with acridine orange or fixed and viewed in Nomarski optics or processed for TUNEL.

EXAMPLE

An assay according to the invention was carried out with staurosporine-pretreated zebrafish embryos, using, as a test compound, a tripeptide inhibitor of ICE-like proteases, carboxybenzoyl Val-Ala-Asp flurorомethylketone (zVADfmk). A control, carboxybenzoyl Phe-Ala-fluoromethylketone (zFAfmk), did not prevent apoptosis in either staurosporine-pretreated embryos, or untreated embryos. The results demonstrated that ICE-like proteases are likely to be involved in mediating apoptosis, within the nervous system and perhaps in other organ systems as well.

CLAIMS

1. A test vertebrate embryo for screening compounds for the ability to affect cell death, said embryo being prepared by the process of:

a) providing a vertebrate embryo, and

b) contacting said embryo with an agent which increases apoptosis in cells of said animal.

2. The embryo of claim 1, wherein said agent is staurosporine.

3. The embryo of claim 1, wherein said vertebrate is a zebrafish.

4. A method of testing a compound for the ability to affect cell death, said method comprising the steps of:

a) providing an animal which, at an embryonic stage, has been contacted with an agent which increases apoptosis in cells of said animal,

b) contacting said animal with said compound, and

c) determining whether said compound affects cell death in said animal.

5. The method of claim 4, wherein step b) is carried out with said animal at an embryonic stage.

6. The method of claim 4, wherein said animal is a vertebrate.

7. The method of claim 6, wherein said vertebrate is a fish.

8. The method of claim 7, wherein said fish is a zebrafish.

9. The method of claim 4, wherein said agent is staurosporine.

10. A method of testing a compound for the ability to affect cell death, said method comprising the steps of:

a) providing an osteichthes embryo which is translucent or transparent,

b) contacting said compound with said embryo, and

c) visually observing the pattern or extent of cell death in said embryo.

11. The method of claim 10, wherein said embryo is a zebrafish embryo.

12. The method of claim 10, wherein cells in said embryo undergoing programmed cell death are labeled in the living embryo for visualization microscopically.

13. The method of claim 12, wherein labelling is carried out by terminal deoxynucleotide transferase dUTP nick labeling.

14. A method for obtaining information on cellular processes, said method comprising the steps of:

a) providing a test and a control zebrafish embryo,

b) subjecting said test embryo to test conditions, and

c) visually observing differences in cells of the test and control embryos, said differences resulting from application of said test conditions.

15. The method of claim 14, wherein said test conditions include mutagenesis-inducing conditions.

16. The method of claim 14, wherein said test and control zebrafish embryos are pre-treated with an agent which affects programmed cell death.

17. The method of claim 16, wherein said agent is a protein kinase inhibitor.

18. The method of claim 17, wherein said protein kinase inhibitor is staurosporine.

19. The method of claim 14, wherein said test conditions include contacting said embryos with a cell death inhibitor.

20. The method of claim 19, wherein said observing includes observing neurons of said embryos, to determine whether neurons in said test embryo which are saved from cell death develop or function normally.

21. The method of claim 19, wherein said cell death inhibitor is a caspase inhibitor.

22. The method of claim 14, wherein the cellular process which is investigated is selected from the group consisting of:

a) neuronal cell function,

b) neuronal connectivity,

c) cell development,

d) tissue development, and

e) organ development.

23. A method of testing a compound for the ability to affect expression of a gene whose expression affects cell death, said method comprising the steps of:

(a) providing an osteichthes test embryo which is translucent or transparent, wherein said gene is expressed in said embryo, wherein said gene either is not normally

expressed in said osteichtheses, or is normally expressed in said osteichthes at a lower level than in said test embryo,

(b) contacting said compound with said embryo, and

(c) visually observing the pattern or extent of cell death in said embryo.

24. The method of claim 23, wherein said osteichthes embryo is a zebrafish embryo.

25. The method of claim 23, wherein said gene is a eukaryotic gene encoding a protein which inhibits cell death.

26. The method of claim 25 wherein said gene encodes bcl-2, and is over-expressed in said embryo.

Abstract of the Disclosure

A test animal for screening compounds for the ability to affect cell death, prepared by the process of: a) providing an animal embryo, and b) contacting the embryo with a protein kinase inhibitor to increase apoptosis in cells of said animal.